Serotonin is involved in the psychostimulant and hypothermic effect of 4-methylamphetamine in rats

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Abstract

4-methylamphetamine (4-MA) has recently emerged as a designer drug of abuse in Europe and it is consumed always with amphetamine. There have been reported some deaths and non-fatal intoxications related to 4-MA. We investigated the changes in locomotor activity and body temperature after 4-MA administration to male Sprague-Dawley rats. Our experiments were carried out at a normal or high ambient temperature. 4-MA (2.5 - 10 mg/Kg, given subcutaneously) increased, in a dose-dependent manner, the horizontal locomotor activity that was significantly reduced by ketanserin, p-cholorophenylalanine (pCPA) or haloperidol. In addition, we have studied the effect of 4-MA on core body temperature by means of an implanted electronic thermograph, enabling continuous measurement of body temperature. We observed a dose-dependent hypothermic response to 4-MA that reached a maximum 45 min after a single injection. We also evidenced slight tachyphylaxis to the hypothermic effect when 4-MA was administered four times in a 2 h interval. The pre-treatment of animals with pCPA or pindolol, but not with ketanserin, fully abolished the hypothermic effect of 4-MA. With all that, we conclude that hypothermia induced by 4-MA is due to the release of 5-HT which activates postsynaptic 5-HT_{1A} receptors.

Keywords: 4-Methylamphetamine, Serotonin, Locomotor activity, Hypothermia, Rat

1. Introduction

4-Methyl-amphetamine (1-(4-methylphenyl)-propane-2-amine; 4-MA) belongs to the group of new psychoactive substances of abuse named and sold as “research chemicals” over the Internet. It seems that 4-MA could be produced accidentally as a result of the use of uncontrolled precursors in the illegal production of amphetamine. The available evidence suggests that, in most cases, 4-MA is sold as amphetamine, and so users are usually unaware of this. When studied as an appetite suppressant agent in the 1950s, 4-MA underwent human clinical trials and the only available study was published in 1952 [1]. However, 4-MA was never made commercially available as a clinically useful drug.

In 2011, 4-MA was found, for the first time, in a seized powder mixture with amphetamine and caffeine in Germany (EMCDDA[1], 2012). Belgium reported six deaths where 4-MA was detected in either post-mortem blood, urine or tissues. In all cases, amphetamine was also present. The concentration of amphetamine in those samples was not very high and, apparently, the cause of death was related to the combined intake of 4-MA and amphetamine. Thereafter, there have been sixteen more deaths and nine non-fatal intoxications related to 4-MA reported in Europe. In 2013, the European Union Council decided an EU-wide control.

The reported human toxicity is most likely the result of the combined dopaminergic activity of amphetamine and the serotonergic activity of 4-MA [2]. In this regard, it was described a massive adrenergic overstimulation just after inhaling material, which had been purchased in the belief that it was amphetamine but also contained 4-MA and dimethyl-amphetamine [3]. Only in one case, a patient with forensic evidence of 4-MA, developed extreme hyperthermia and died after cardiac arrest, although he had consumed ketamine, cocaine and other amphetamines 24 h before (EMCDDA-Europol report 2012).

There are very few published studies on the pharmacology of 4-MA in laboratory animals [4,5]. It has been speculated that, compared to amphetamine, the most pronounced serotonergic action of 4-MA could diminish the psychoactive effects of amphetamine leading to the consumption of repeated and higher doses of contaminated amphetamine [2].

[1] European Monitoring Centre for Drugs and Drug Addiction. Technical report on 4-methylamphetamine.

Serotonin (5-HT) in the hypothalamus is involved in the regulation of body temperature [6]. Stimulation of 5-HT_2 receptors in hypothalamic neurons mediates hyperthermic effects, whereas stimulation of the 5-HT_{1A} receptors in these neurons inhibit the endogenous release of hypothalamic 5-HT and leads to hypothermic effects [7,8]. It is well known that the effects of amphetamine on body temperature are biphasic and, although low doses induce decreases in body temperature, higher doses produce vigorous thermogenesis [9,10]. To our knowledge, there is no study about the effects of 4-MA on body temperature.

Because the main problem associated to 4-MA exposure is the acute toxicity of this drug, which seems to be linked to hyperthermia, the aim of the present study was to characterize 4-MA as regards to its ability to induce a psychostimulant effect and, what is more important, to alter body temperature in rats. We focused our attention on 5-HT system. This represents a first approach to characterize the pharmacology of 4-MA in rats and hypothesizes the translation of its effects to human abusers.

2. Materials and methods

2.1. Animals and treatments

Male Sprague-Dawley rats (125 – 175 g) (Janvier, Lé Genest, France) were used. The animals were housed in a regulated ambient temperature with a 12 h light/dark cycle (lights on at 08:00 h) with free access to food and water. Saline and 4-MA were administered subcutaneously. Ketanserin, haloperidol and pindolol were dissolved in saline and administered intraperitoneally 20 min before saline or 4-MA. Treatment with *p*-chlorophenylalanine (pCPA) was carried out at a dose of 300 mg/Kg (i.p.) given daily, over the course of three days. Eighteen hours after the last dose of pCPA, the animals received saline or 4-MA. Experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia, and following the guidelines of the European Communities Council (86/609/EEC).

2.2. Synthesis of 4-MA

We synthesized 4-MA following the procedure previously described [11] with some modifications. Briefly, 4-methylphenyl-2-nitropropene precursor was obtained by the classical Knoevenagel condensation between *p*-tolualdehyde and nitroethane in acetic acid/ammonium acetate. The mixture was heated at 50 - 60 ºC for 2 h and the reaction was stopped with cold water. The yellow solid was filtered and recrystallized from ethanol/hexane (1:1). The 4-methylphenyl-2-nitropropene was dissolved in

tetrahydrofuran (THF) and lithium aluminium hydride solution (1M) in THF was added drop wise. The mixture was allowed to stir overnight at room temperature. Excess hydride was decomposed by the cautious addition of H_2SO_4 (8%). Diethylether was added and the aqueous layer was extracted and cleaned with potassium sodium tartrate. The aqueous layer was made alkaline using NaOH (5%) and the amine was extracted with dichloromethane. Finally, the free base was converted to the hydrochloride salt using a solution of hydrogen chloride (2M) in diethylether. The identification of 4-MA was assessed by ^{1}H NMR) (d_6-DMSO) yielding the following results: δ 8.32 (s, 3H); 7.13 (d, 2H) J=8.4 Hz; 7.10 (d, 2H) J=8.4 Hz; 3.32 (m, 1H); 2.99 (dd, 1H) J=13.4, 5.2 Hz; 2.59 (dd, 1H) J=13.4, 9.2 Hz; 2.27 (s, 1H); 1.08 (d, 3H) J=6.4 Hz. Chemical purity of the obtained compound was also assessed by melting point determination, thin layer chromatography and mass spectrometry. All analytical data were consistent with the assigned structure with over 98% purity for the 4-MA.

Isoflurane was from Lab Dr. Esteve (Barcelona, Spain). All other drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO,USA).

2.3. Spontaneous locomotor activity

Prior to experimentation, all rats received two habituation sessions of manipulation (48 and 24 h before testing) whose purpose was to reduce the novelty and stress associated with handling and injection. During these sessions, animals were placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets of 16 infrared photocells (LE8811, PANLAB, SL, Barcelona, Spain) mounted according to the x, y axis coordinates and 1.5 cm and 7.5 cm above the wire mesh floor. Occlusions of the photo beams were recorded and sent to a computerized system (SedaCom32, PANLAB, SL, Barcelona, Spain). The interruption counts of the lower level, over a 10 min-slot, were used as a measure of horizontal locomotor activity. Similarly, interruption counts of the upper level were used as a measure of rearing. On the day of testing, the animals were placed in the activity box and received the drug treatment 30 min after. Results are expressed as AUC (area under the curve), which was measured as the total changes from baseline at each recording interval over 150 min.

2.4. Surgical procedures and continuous acquisition of body temperature

The animals were allowed 1 week after arrival to acclimatize before surgery. Subsequently, they had implanted an electronic thermograph (Thermo Tracker, Barcelona, Spain), enabling continuous measurement of core body temperature. The implant was placed in the abdominal cavity as follows: the rats were anesthetized with

isoflurane, the abdomen was opened by making a 2-cm midline incision and the device was placed in the abdominal cavity, along the sagittal plane. The abdominal and the skin wound were then closed with absorbable suture material. After surgery, animals were individually housed, received analgesic therapy and were allowed to recover for 7 days before saline or 4-MA administration. The device registered the core temperature every 5 min and the values were downloaded to a computer after removal of the device, once the animals had been sacrificed, using the interface and software provided by the manufacturer. Data were acquired from 2 h prior to until 12 h after drug administration.

2.5. Statistical analyses

Data were analysed using one way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons, or Student-t test when appropriate, using GraphPad Instat (GraphPad, San Diego, CA). The difference between treatments was considered statistically significant at $p < 0.05$. All results were expressed as mean ± S.E.M.

3. Results

3.1. Effect of 4-MA on spontaneous locomotor activity

An overall ANOVA demonstrated an extremely significant effect of 4-MA treatment (2.5 -10 mg/Kg) in the increase of horizontal locomotor activity in rats ($F_{3,14} = 16.872$, $p < 0.001$). This increase was dose-dependent (Fig. 1).

Except for the lowest dose, the duration of the hyperlocomotive effect was of 120 min. In addition to our analysis of locomotor activity, we simultaneously recorded the number of rearings as a measure to determine the habituation of animals to the novel environment. The animals treated with different doses of 4-MA showed no difference with saline group in this behaviour ($F_{3,14} = 2.233$, n.s.).

In a separate group of experiments, we monitored the locomotor response to 4-MA (2.5mg/kg), at normal (21 ± 1 ºC) and high (26 ± 1 ºC) ambient temperature. No differences were found (AUC values: 50850 ± 1420, at normal and 46411 ± 1420 at high ambient temperature).

3.1.1. Involvement of 5-HT and DA in the psychostimulant effect of 4-MA

A series of experiments with animals pre-treated with different compounds were carried out to better understand the mechanism responsible of the increase of locomotor activity induced by 4-MA (administered at a dose of 2.5 mg/Kg). To prove the involvement of 5-HT we pre-treated animals with a dose of ketanserin (1 mg/Kg), an antagonist of 5-$HT_{2A/2C}$ receptors, that did not affect the basal locomotor activity. This compound inhibited partially the effect of 4-MA. Additionally, the pre-treatment of animals with pCPA, an inhibitor of 5-HT synthesis (3 x 300 mg/kg), significantly reduced the effect of 4-MA. Furthermore, to prove the involvement of dopamine in the increase of locomotor activity induced by 4-MA we pre-treated animals with the maximum dose of haloperidol (0.1 mg/Kg), an antagonist of dopamine receptors, which did not affect the locomotor activity. This compound inhibited partially the effect of 4-MA (Fig. 2).

3.2. Effect of 4-MA on body temperature

3.2.1. Effect of a single dose of 4-MA at normal and high ambient temperature

In experiments performed at a normal (21 ± 1 ºC) ambient temperature, we observed a strong but transient dose-dependent hypothermic response that persisted until 90-100 min after the administration of 4-MA. When the curve profile analysis was carried out, it showed a maximum drop in body temperature between 40 and 45 min after drug administration (Fig. 3). What is more, saline-treated rats exhibited an overall non-significant (p = 0.085) increase in temperature after 4-MA administration, probably due to the stress induced by the injection.

It has been argued that amphetamines' toxicity may be potentiated by the overheated environments as they exacerbate one of the most toxic effects of the drug, the impairment of thermoregulation [12]. To this end, we have studied the effect of 4-MA in a high temperature environment (26 ± 1 ºC). When experiments were performed at this temperature, the hypothermic response induced by 4-MA also took place ($F_{2,16}$ = 8.566, $p < 0.01$), (saline: 37.73 ± 0.21, n = 5; 4-MA 2.5 mg/Kg: 36.25 ± 0.29, n = 6, $p <$ 0.05 vs saline; 4-MA 5 mg/Kg: 35.79 ± 0.43, n = 6, $p< 0.01$, vs saline). The lowest body temperature was observed at 90-100 min after drug administration. Notice that the maximum drop in body temperature appeared later than that observed at 21 ºC.

3.2.2. Effect of repeated doses of 4-MA .

It is known that some tolerance or tachyphylaxis to the hyperthermic effect of certain psychostimulant drugs [13] can exist. We administered 4-MA at three different

doses (2.5, 5 and 7.5 mg/Kg) four times in a 2 h interval in a normal ambient temperature. As can be seen in Table 1, all administered doses induced hypothermia. Tachyphylaxis to this effect was only detected after the fourth administration because the maximum of hypothermia (at both fourth doses of 5 and 7.5 mg/Kg) differed from the first one ($p < 0.05$).

3.2.3. Effect of blockade of 5-HT synthesis

As previously stated, we investigated the role of endogenous 5-HT on the effect of 4-MA on body temperature. pCPA was administered in the same schedule previously described for locomotor activity experiments. The obtained results (saline: 37.75 ± 0.24 ºC, n = 5; 4-MA 2.5 mg/Kg: 35.96 ± 0.37 ºC, n = 5, $p < 0.05$, vs saline; pCPA: 36.90 ± 0.49 ºC, n = 5; pCPA + 4-MA 2.5 mg/Kg: 37.18 ± 0.30 ºC, n = 5), demonstrated that the blockade of 5-HT synthesis fully abolished the hypothermic effect of 4-MA.

3.2.4. Effect of 5-HT_{1A}/5-HT_2 receptor blockade

Animals were pre-treated with pindolol (2 mg/Kg) before 4-MA (2.5 mg/Kg). Pindolol fully abolished the hypothermic effect of 4-MA.

Obtained results were as follows: saline: 37.41 ± 0.26 ºC, n = 5; 4-MA 2.5 mg/Kg: 35.79 ± 0.46 ºC, n = 5, $p < 0.05$, vs saline; pindolol: 37.70 ± 0.23 ºC, n = 4; pindolol + 4-MA 2.5 mg/Kg: 37.13 ± 0.56 ºC, n = 4). On the contrary, pre-treatment of animals with ketanserin (1 mg/Kg) did not inhibit the hypothermia induced by 4-MA (saline: 37.76 ± 0.20, n = 5; 4-MA 2.5 mg/Kg: 35.95 ± 0.31, n = 5, $p < 0.05$, vs saline; ketanserin: 36.96 ± 0.29, n = 3; ketanserin + 4-MA 2.5 mg/Kg: 35.96 ± 0.37, n = 5, $p < 0.05$, vs saline). Consequently, the blockade of 5-HT_{1A} (not 5-HT_2) receptors abolished the hypothermic effect of 4-MA.

4. Discussion

Originally developed as an appetite suppressant, 4-MA has recently raised again as a new psychoactive substance in Europe, and it can be found as a drug of abuse, always mixed with amphetamine [14]. 4-MA can be produced at a relatively low cost and the precursors known to be used for its manufacture are not under control and appear to be commercially available. It is unclear to what extent 4-MA is advertised/sold via the Internet. The observed toxicity in humans is most likely the result of the combined dopaminergic activity of amphetamine and the serotonergic activity of 4-MA. Also, slow metabolism of 4-MA and its MAO-inhibiting properties can also contribute to the toxicity of 4-MA [14].

There are very few published studies on the pharmacology of 4-MA. This compound is equipotent as a releaser of dopamine, norepinephrine and 5-HT [4], It also shows low affinity for 5-HT_2 receptors [15]. However, with respect to 5-HT release, 4-MA was significantly more potent compared to amphetamine [4].

Studies of the behavioural actions of amphetamines commonly focus on locomotion. Wellman et al. compared the psychostimulant effect of three amphetamine analogues, including 4-MA [5]. They concluded that 4-MA had a very low effect on locomotion that is attributed to a high activity at 5-HT transporter. In that study, the range of doses used was up to 2.4 mg/Kg and locomotion was registered over three 15 min periods (15, 30 and 45 min after injection).

In the present study, the hyperlocomotion induced by 4-MA was dose-dependent and already present at the dose of 2.5 mg/Kg. This effect was not affected by increasing the ambient temperature. There is limited information available from user reports or Internet discussion forums about 4-MA dosage. The only information is from an abuser that self-reported use of 4-MA by oral (160 mg) and intramuscular (80–120 mg) route (EMCDDA, 2012). The human equivalent doses of the range used (2.5 – 10 mg/Kg) in the present study, although given subcutaneously, are 30 – 120 mg [16], which are in agreement with those reported as psychostimulants.

It is known that amphetamine derivatives, like MDMA, produce an increase in the dopamine release by activating 5-HT_2 receptors, which may also contribute to the hyperlocomotion [17]. In present experiments, when animals were pre-treated with ketanserin or haloperidol (at doses that did not affect basal locomotor activity), the hyperlocomotion of 4-MA was partially inhibited, suggesting both 5-HT and dopamine take part in this effect. According to previously published studies [15] ruling out a direct activation of 5-HT_2 receptors by 4-MA, we hypothesize that this drug promotes the release of 5-HT, which can activate 5-HT_2 receptors, inducing an increase of synaptic dopamine concentration that finally activates dopamine receptors and contributes to hyperlocomotion. The inhibition after pCPA treatment demonstrates the role of endogenous 5-HT in the hyperlocomotion induced by 4-MA. Furthermore, 4-MA-treated rats did not experience the environment as a novel one (absence of significant difference in rearings from saline-treated animals),

The main problem associated to 4-MA exposure is the acute toxicity of this drug, which seems to be linked to hyperthermia. For this reason, we focused our attention on the effects of 4-MA on body temperature. We have studied it after an acute dose of 4-MA but, in the effort to mimic recreational use, we also considered appropriate to

simulate the widespread practices of “boosting or redosing” (taking supplemental doses over time in order to maintain the drug effect, hence becoming dependent to achieve the ‘high’ effect). For this reason, we chose to administer multiple doses of 4-MA during a daily session, with an interval of 2 h between doses [18].

In our experiments, 4-MA induced a consistent hypothermic response that persisted for 90-100 min, in all the doses assayed. Green et al. studied the effect of MDMA on body temperature and found that its administration to rats produces hyperthermia if animals are housed in normal or warm ambient room temperature conditions (⩾20°C), but hypothermia when in cool conditions (⩽17°C) [19]. Perhaps the difference between both drugs is that the potency and selectivity of 4-MA as serotonin releaser is higher than other amphetamine derivatives [4]. A slight tachyphylaxis to this effect was found because after a repeated administration schedule, the maximum of hypothermia after the fourth administration significantly differed from the first one.

It has been described that local perfusion of the hypothalamus with 5-hydroxytryptophan (a 5-HT precursor) or fluoxetine (a 5-HT reuptake inhibitor) increases body temperature by increasing extracellular concentrations of 5-HT, which activate central 5-HT_{2A} receptors [20,21], although activation of these receptors in the peripheral system leads to hypothermia [22]. Additionally, an activation of 5-HT_{1A} receptors in the hypothalamus leads to hypothermia [23].

In our experiments, 4-MA induced hypothermia was not abolished by a 5-$HT_{2A/2C}$ receptor antagonist. Additionally, results from pindolol and pCPA-treated animals in which the hypothermic effect of 4-MA was significantly reduced, lead us to conclude that hypothermia induced by 4-MA can be the consequence of 5-HT release that activates postsynaptic 5-HT_{1A} receptors [23].

5. Conclusions

To sum up, we have shown that 4-MA, at doses that imitate human recreational use, induces an increase on the horizontal locomotor activity in rats, involving both 5-HT and dopamine. Moreover, at the same doses, 4-MA induces dose-dependent hypothermia by an activation of postsynaptic 5-HT_{1A} receptors. These results make it very unlikely that the only event of hyperthermia described in humans could be due to a direct effect of 4-MA. Overall, this study states as the first approach to characterize the pharmacology of 4-MA in rats and contributes to our knowledge of this new drug of abuse.

Conflicts of interest

None.

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Legends of figures

Fig. 1. Effect of a single subcutaneous administration of 4-MA (2.5, 5 or 10 mg/Kg) and saline. For locomotor activity, the interruption counts in the lower frame of the apparatus were registered, displayed in a 10 min-slot and monitored for 150 min. Data are expressed as the mean ± SEM of values from 4 animals. *$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ vs saline.

Fig. 2. Effect of ketanserin (Ket) (1 mg/Kg), haloperidol (Hal) (0.1 mg/Kg) and pCPA (3 x 300 mg/kg) on the increase of locomotor activity induced by 4-MA (2.5 mg/Kg). Data (AUC, Area under the Curve) are expressed as the mean ± SEM of values from 5 animals. *$p < 0.05$ and ** $p < 0.01$ vs 4-MA.

Fig 3. Effect of 4-MA on core body temperature. Rats (n=5 per group) were treated with saline (1ml/Kg, s.c.) or 4-MA (1, 2.5, 5 or 10 mg/Kg, s.c.) and core body temperature was recorded using an electronic implant at 5 min-intervals for 12 h. Results are presented as group means. All SEM values are omitted for the sake of clarity. Injection time is indicated by an arrow.

Figure 1

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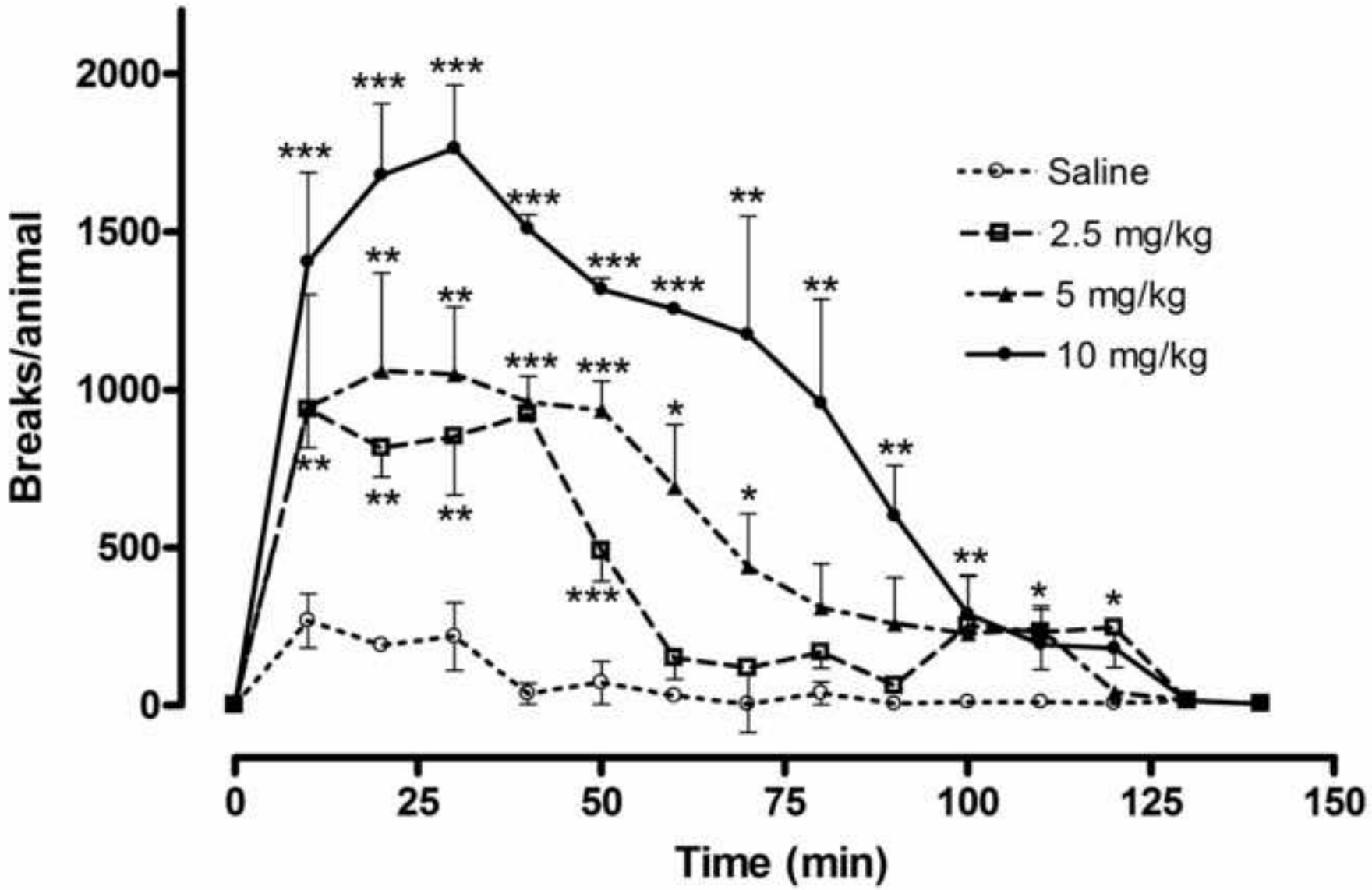

Figure 2

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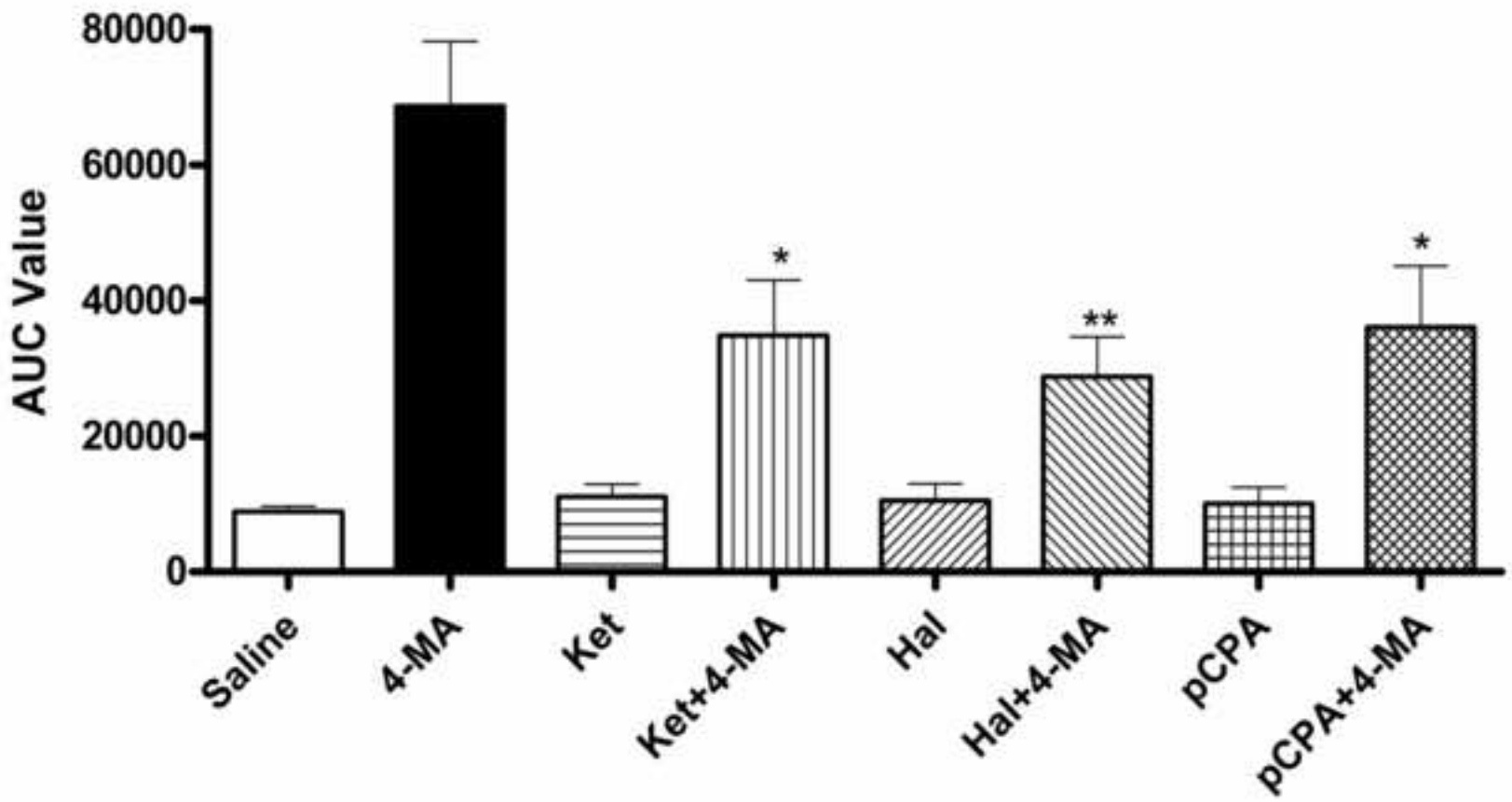

Figure 3

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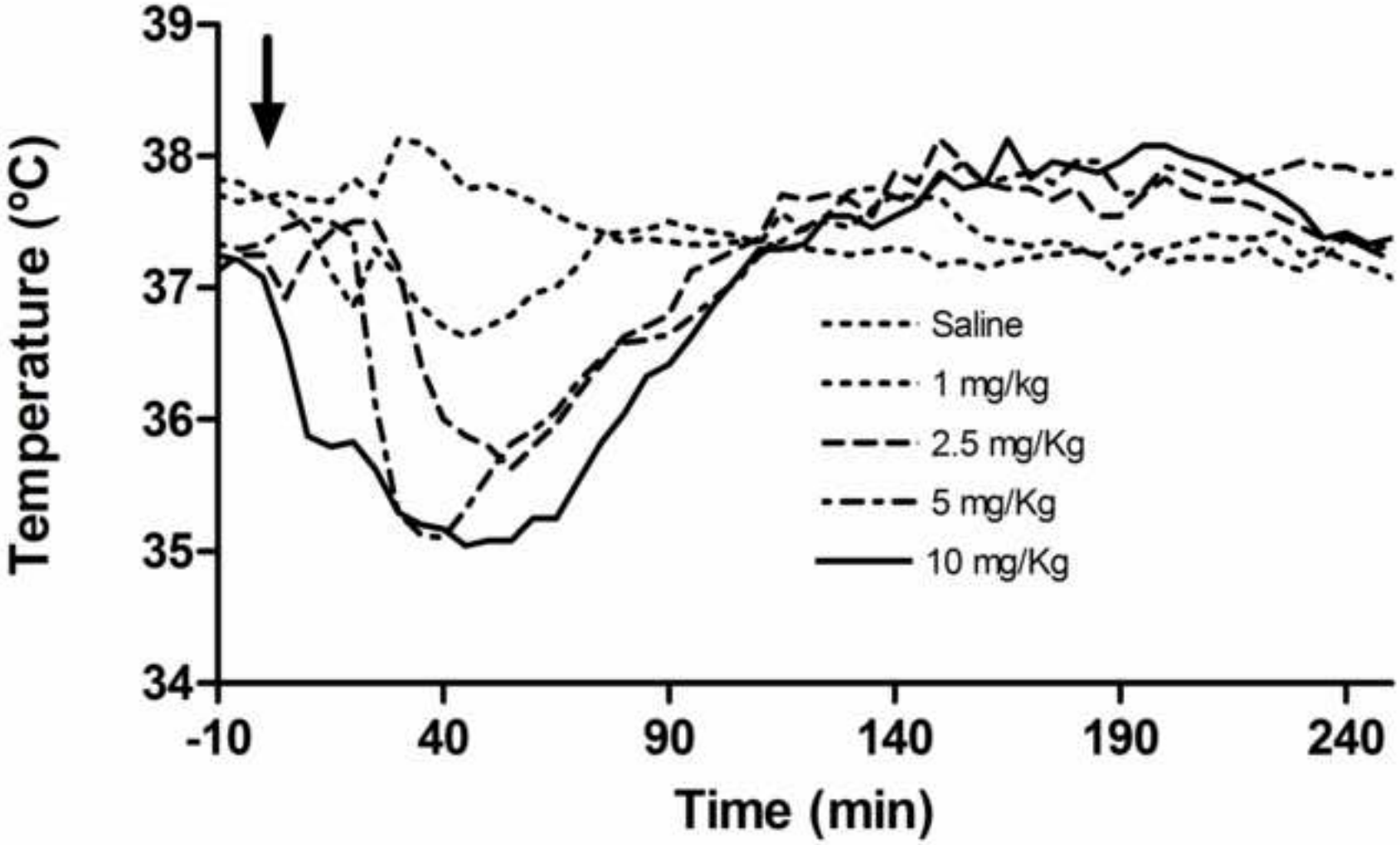

Table 1

Table 1. Effect of repeated doses, at 2 h interval, of 4-MA on body temperature. Results denote the maximum of hypothermia and the time after each injection in which this maximum is achieved. Results are expressed as mean ± S.E.M. from four animals. * $p < 0.05$ versus the first dose.

	Dose 1		Dose 2		Dose 3		Dose 4	
Drug	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Temp (°C)	Time (min)
Saline	37.87 ± 0.26	----	37.71 ± 0.19	----	37.44 ± 0.17	----	37.21 ± 0.16	----
4-MA (2.5 mg/Kg)	36.09 ± 0.27	45	35.06 ± 0.28	40	35.31 ± 0.18	45	36.31 ± 0.80	40
4-MA (5 mg/Kg)	34.31 ± 0.31	50	34.63 ± 0.29	60	35.44 ± 0.38	50	35.97 ± 0.40*	45
4-MA (7.5 mg/Kg)	33.54 ± 0.29	75	33.88 ± 0.82	60	34.00 ± 0.63	55	34.63 ± 0.27*	65